Slk1 is a meiosis-specific Sid2-related kinase that coordinates meiotic nuclear division with growth of the forespore membrane

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Accepted 4 February 2008
Journal of Cell Science 121, 1383-1392 Published by The Company of Biologists 2008
doi:10.1242/jcs.023812

Summary

Septation and spore formation in fission yeast are compartmentalization processes that occur during the mitotic and meiotic cycles, and that are regulated by the septation initiation network (SIN). In mitosis, activation of Sid2 protein kinase transduces the signal from the spindle pole body (SPB) to the middle of the cell in order to promote the constriction of the actomyosin ring. Concomitant with ring contraction, membrane vesicles are added at the cleavage site to enable the necessary expansion of the cell membrane. In meiosis, the forespore membrane is synthesized from the outer layers of the SPB by vesicle fusion. This membrane grows and eventually engulfs each of the four haploid nuclei. The molecular mechanism that connects the SIN pathway with synthesis of the forespore membrane is poorly understood. Here, we describe a meiosis-specific Sid2-like kinase (Slk1), which is important for the coordination of the growth of the forespore membrane with the meiotic nuclear divisions. Slk1 and Sid2 are required for forespore membrane biosynthesis and seem to be the final output of the SIN pathway in meiosis.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/9/1383/DC1

Key words: Meiosis, Forespore membrane, Sporulation, SIN, Sid2, Slk1

Introduction

Membrane biosynthesis needs to be highly coordinated with cell division. During mitosis, the plasma membrane of the mother cell is inherited by the two daughter cells. By contrast, in meiosis, the formation of germ cells requires the de novo synthesis of plasma membranes within the mother-cell cytoplasm. In fission yeast, this process is called sporulation and is initiated during the second nuclear division by the formation of a double-layered membrane, termed the forespore membrane. Synthesis of the forespore membrane is closely related – both temporally and spatially – to the second meiotic nuclear division and is initiated from the spindle pole bodies (SPBs). In meiosis II, at the metaphase-to-anaphase transition, SPBs undergo a transient change in shape from a dot into a crescent (Hagan and Yanagida, 1995). The cytoplasmic face of SPBs differentiates into multilayered plaques (Hirata and Shimoda, 1994; Shimoda and Nakamura, 2004; Tanaka and Hirata, 1982); the inner side of these plaques forms the meiotic spindle, whereas their outer side serves as a platform for the assembly of the forespore membrane (Hirata and Shimoda, 1994; Tanaka and Hirata, 1982). The forespore membrane grows by vesicle fusion and eventually encapsulates each of the four haploid nuclei. Finally, spore walls are synthesized by the accumulation of wall materials – lipids and polysaccharides – between the inner and outer membranes of the forespore (Hirata and Shimoda, 1994; Tanaka and Hirata, 1982).

The septation initiation network (SIN) is an SPB-associated signal-transduction pathway that regulates cytokinesis during the mitotic cell cycle. The SIN consists of the Spg1 GTPase; the downstream kinases Cdc7, Sid1 and Sid2; and the associated proteins Cdc14 (in complex with Sid1) and Mob1 (in complex with Sid2) (Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Salimova et al., 2000; Schmidt et al., 1997; Sparks et al., 1999). Sid4 and Cdc11 form an assembly platform for the SIN components at the SPB (Chang and Gould, 2000; Krapp et al., 2001; Tomlin et al., 2002). Activation of Sid2 is the key output of the SIN pathway that presumably transmits the signal from the SPBs to the medial ring, where it activates actomyosin-ring contraction and septation (Sparks et al., 1999).

The role of the SIN in meiosis, in which the cell does not form a contractile ring or a division septum, has only been clarified recently (Krapp et al., 2006). Most SIN genes, with the interesting exception of *sid2*, are highly transcribed during meiosis, peaking at meiosis II (Mata et al., 2002). Mutants in SIN components can complete the meiotic nuclear divisions but cannot form spores. Therefore, the SIN pathway seems to be essential for proper forespore membrane formation around the haploid nuclei (Krapp et al., 2006).

Here, we describe Slk1, which is a Sid2 paralogue that is only expressed in meiosis, in which it is required to couple the growth of the forespore membrane to the meiotic nuclear division. We propose that Slk1, together with Sid2, could be the main output of the SIN pathway in meiosis.

Results

slk1 is a meiosis-specific *sid2* paralogue

The *slk1* (*mug27*) gene was identified in a large-scale-deletion screening of meiotically upregulated genes (Martin-Castellanos et al., 2005). Viable *mug* mutants (167 in total) were systematically

analyzed after mating and sporulation for spore number and morphology, as well as for the number and relative size of the nuclei after DAPI staining. Deletion of *mug27*, also known as *ppk35*, showed asci with abnormally small spores (Martin-Castellanos et al., 2005) (Fig. 3A). The *mug27* gene encodes a paralogue of Sid2, a conserved serine-threonine protein kinase that belongs to the Ndr subfamily of the AGC group of kinases (Tamaskovic et al., 2003) (Fig. 1A). Dbf2 and Dbf20 in *Saccharomyces cerevisiae*, Ndr1 and Ndr2 in metazoans, and Orb6 in *Schizosaccharomyces pombe* are members of this group of kinases (Fig. 1B,C). Accordingly, we renamed Mug27 as Slk1, for Sid2-like kinase 1.

We studied the expression of *slk1* in the diploid strain *h⁻/h⁻ pat1-114/pat1-114*, containing a thermosensitive mutation for the *pat1* gene. Exponentially growing cells (Fig. 2, exp) were pre-synchronized in G1 by nitrogen starvation at 25°C for 14 hours (Fig. 2, *t*=0 hours). Nitrogen was reintroduced and the cultures were incubated at 34°C to inactivate the Pat1 kinase (Bahler et al., 1991). Under these conditions, the cells underwent a synchronous meiosis (Fig. 2C,D): *slk1* mRNA was not transcribed in exponentially growing cells; it was expressed during prophase and peaked between meiosis I and meiosis II, according to the microarray expression data (Mata et al., 2002) (Fig. 2A). To analyze Slk1 protein levels, we constructed the diploid strain *h⁻/h⁻ pat1-114/pat1-114 slk1-GFP/slk1-GFP*. These cells contain a functional version of Slk1 tagged at the C-terminus with the green fluorescent protein (GFP). Slk1 protein levels followed those of the mRNA and remained high up to the second meiotic nuclear division (Fig. 2B). This experiment indicates that *slk1* mRNA and protein are meiosis-specific.

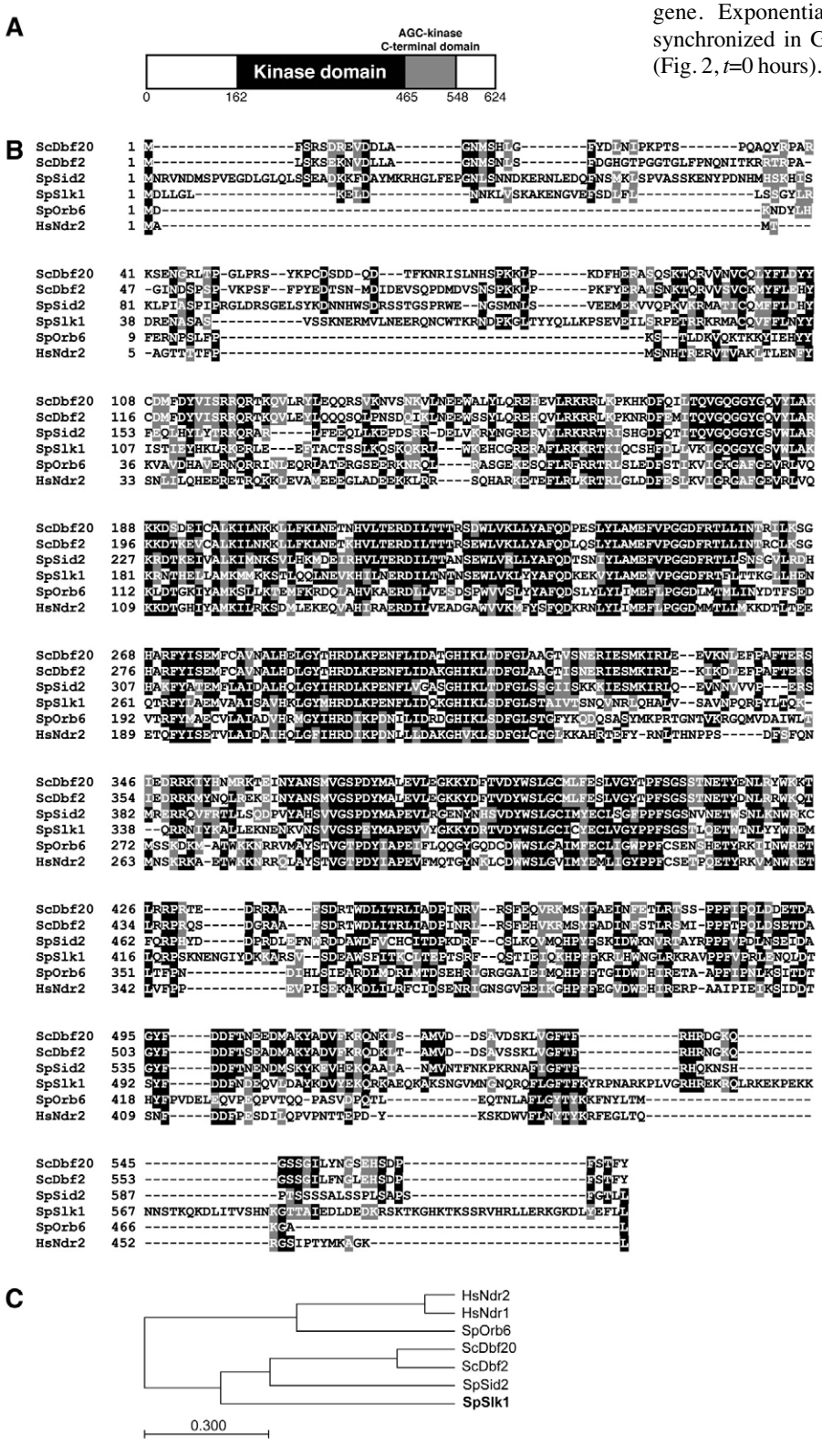

Fig. 1. *slk1* encodes a serine-threonine protein kinase similar to *S. pombe* Sid2. (A) Schematic representation of the Slk1 domains. The kinase domain is represented in black and the AGC-kinase C-terminal domain is represented in grey. The amino acid positions of these domains are indicated. (B) Sequence comparison of Slk1 and related proteins. Sequence alignment was generated using CLC Free Workbench 4.0.3 software (CLC Bio, Aarhus, Denmark). Shading was performed with the Boxshade 3.21 program at the http://www.ch.embnet.org/ server. Identical amino acids are highlighted in black and similar amino acids are highlighted in grey. Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*. (C) The phylogenetic tree was constructed using CLC Free Workbench 4.0.3 software according to the UPGMA algorithm.

The *slk1* mutant is defective in spore formation

To investigate the function of Slk1, the *slk1* deletion was examined. Haploid cells deleted for *slk1* (*slk1Δ*) showed no apparent growth or cell cycle defects (Fig. S1A,B in the supplementary material) and they were able to mate with the same efficiency as wild-type cells (data not shown), suggesting that *slk1* has no obvious function in the mitotic cell cycle. This is consistent with the fact that *slk1* is expressed only during meiosis.

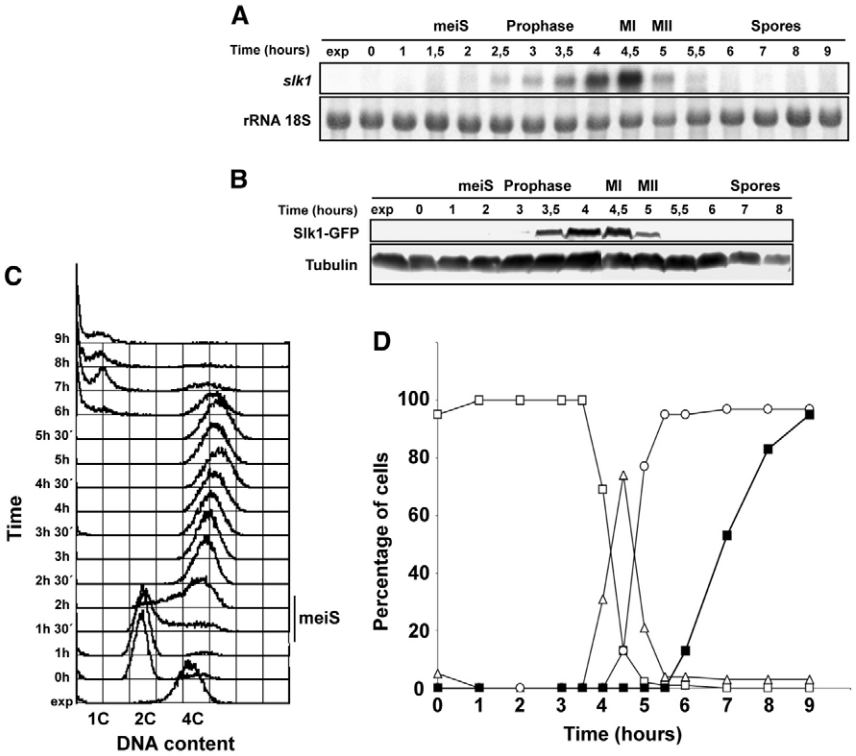

Fig. 2. *slk1* expression is meiosis-specific. (A) Northern-blot analysis of *slk1* expression in a *pat1*-driven meiosis. Strain h^{-}/h^{-} *pat1-114/pat1-114* (S964) was induced to enter synchronous meiosis and total RNA was extracted at the indicated times. Samples were processed and blotted, and filters were hybridized with a *slk1* probe. As a loading control, 18S rRNA stained with Methylene Blue is shown. exp, exponential growth; meiS, pre-meiotic S-phase; MI, meiosis I; MII, meiosis II. (B) The same experiment as in A, but in this case strain h^{-}/h^{-} *pat1-114/pat1-114 slk1-GFP/slk1-GFP* (S1706) harbouring a functional version of *slk1* tagged with GFP was used, and protein was extracted at the indicated times and processed for western blot. Slk1-GFP was detected with anti-GFP antibodies. Tubulin levels are shown as a loading control. (C) FACS analysis of samples taken during the experiment shown in A. Pre-meiotic S-phase occurs between 1.5 and 2 hours. Spores generated are released during sample processing, and appear as a 1C peak between 6 and 9 hours. (D) Meiosis progression of the experiment shown in A was monitored by DAPI staining. White square, one nucleus; triangle, two nuclei (meiosis I); circle, three to four nuclei (meiosis II); black square, spores. At least 300 cells were counted for each time-point.

To study the function of Slk1 in meiosis, homothallic h^{90} *slk1Δ* mutants were mated. In fission yeast, mating is followed by meiosis and sporulation. These cells were able to complete both meiotic nuclear divisions and showed four spores or less, which were smaller than those of the wild-type control (Fig. 3A). The spore viability of four-spore asci was analyzed by tetrad dissection, and no differences were observed between wild-type and *slk1Δ* cells (data not shown). To analyze meiotic cell cycle progression in the *slk1* mutant, we induced synchronous meiosis in *pat1-114* strains. As shown in Fig. 3B, *slk1Δ* cells proceed through meiotic divisions with kinetics identical to that of the wild type.

Sporulation in *S. pombe* requires the de novo synthesis of plasma membrane within the mother-cell cytoplasm. This process is initiated during meiosis II, at the metaphase-to-anaphase transition, from the cytoplasmic face of the SPBs by the formation of a double-layered membrane, termed the forespore membrane (Shimoda, 2004; Shimoda and Nakamura, 2004). Psy1 is a t-SNARE syntaxin 1A that localizes to the plasma membrane in vegetative cells and that, in meiosis, relocalizes to the nascent forespore membrane (Nakamura et al., 2001), where presumably it is required for vesicle fusion. The forespore membrane can be visualized by using the fusion protein Psy1-GFP. In metaphase II, Psy-GFP staining is shaped as an arc that then develops into a cup-like structure by extension of the membrane, which eventually closes to form a double-layered

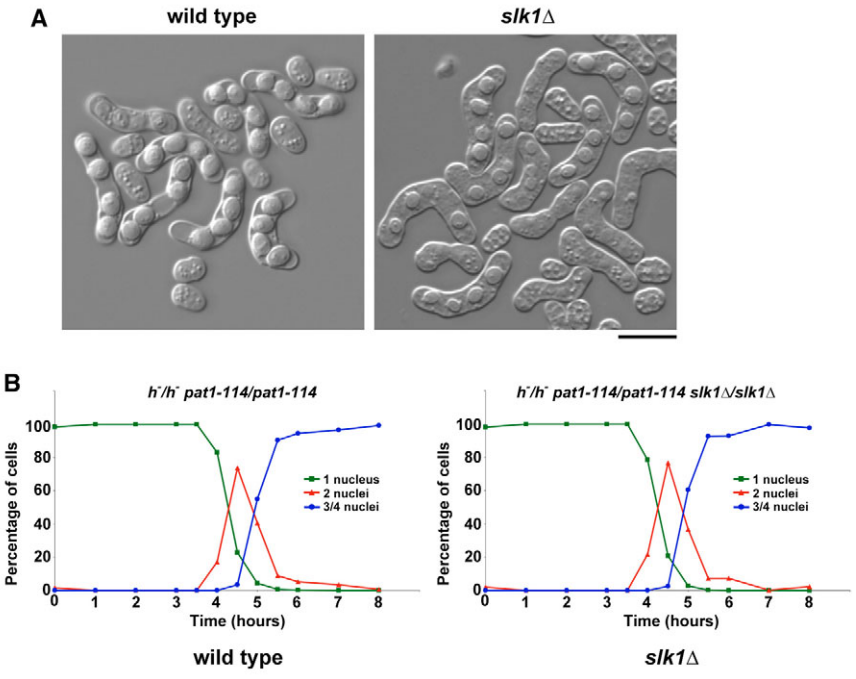

Fig. 3. *slk1* is required for spore formation. (A) The *slk1Δ* mutant produces asci with small spores. Wild-type (S1478) and *slk1Δ* (S1883) h^{90} homothallic strains were incubated on sporulation media (MEA) for 2 days, and Nomarski microphotographs were taken. Scale bar: 10 μm. (B) The *slk1Δ* mutant proceeds through meiotic divisions with normal kinetics. Meiosis progression in *pat1-114* diploid strains S964 (wild type) and S1566 (*slk1Δ*) was monitored by DAPI staining. Percentages of cells with one nucleus (green square), two nuclei (red triangle; meiosis I) and three to four nuclei (blue circle: meiosis II) are shown.

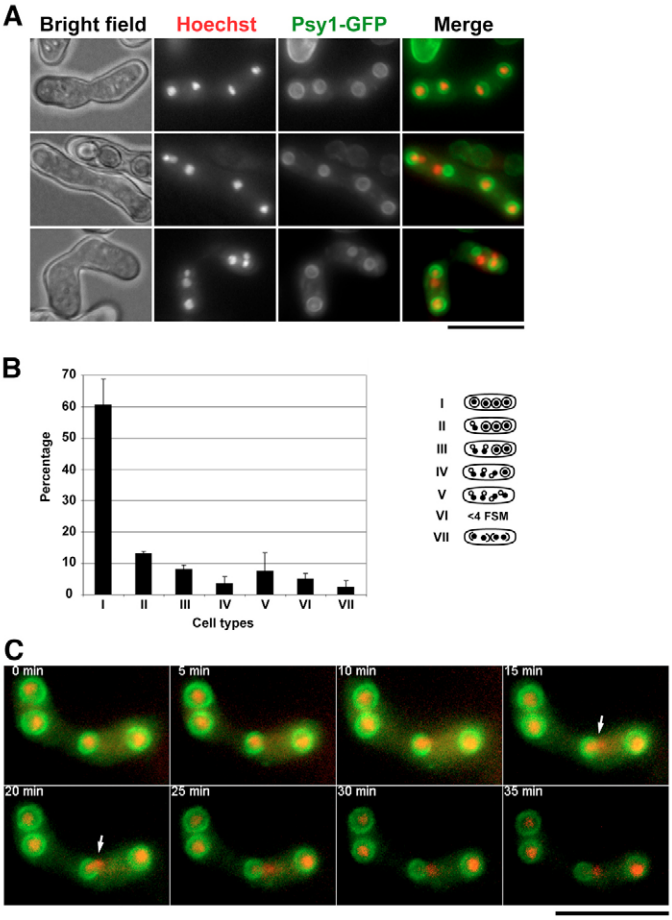

Fig. 4. *slk1* is required for proper engulfment of the nuclei by the forespore membrane at the end of meiosis. Homothallic h^{90} strains S1478 (wild-type) and S1883 (*slk1Δ*) carrying the plasmid pREP81-Psy1-GFP were sporulated on MEA plates at 25°C. (A) A percentage of *slk1Δ* cells show defects in nucleus engulfment by the forespore membrane, as revealed by Psy1-GFP fluorescence. Three representative cells are shown. Left panel: bright-field images. Central panels: Hoechst and GFP fluorescence. Right panel: merge. (B) Frequency of abnormal forespore membrane formation in the *slk1Δ* mutant. Cells are classified according to the number of defective spores. In class I, all nuclei are engulfed by the forespore membranes; class II, one forespore membrane fails to engulf a nucleus; class III, there are two defective forespore membranes; class IV, there are three defective forespore membranes; class V, there are four defective forespore membranes; class VI, there are fewer than four forespore membranes; and class VII, forespore membrane formation is incomplete. Means and standard deviations of three independent experiments are presented. In each experiment, at least 200 cells were counted. (C) Time-lapse experiment showing forespore membrane growth in a *slk1Δ* strain (S1883) carrying the plasmid pREP81-Psy1-GFP. Cells were sporulated on MEA plates and DNA was stained with Hoechst. Stacks of five images separated by 1 μm were taken every 5 minutes. GFP (green) and Hoechst (red) merged images were generated with ImageJ. Arrows mark the abnormal engulfment of DNA by the forespore membrane in one spore. Scale bars: 10 μm.

membrane containing one haploid nucleus generated during meiosis II and a small amount of cytoplasm. We decided to examine the phenotype of *slk1Δ* mutant spores in greater detail by expressing Psy1-GFP and staining the nuclei with Hoechst (Fig. 4A). As shown in Fig. 4B, 60% of the asci showed four nuclei surrounded by the forespore membrane; the rest contained three, two, one or no nuclei encapsulated by the forespore membrane, suggesting a role of Slk1 in the growth of the forespore membrane and engulfment of the nuclei. In the *slk1* mutant, the forespore membrane initiated growth around the four nuclei but in some cases failed to engulf one or more nuclei (Fig. 4C, arrow). A closer examination of forespore membrane formation in wild-type and *slk1Δ* cells by video microscopy revealed that the initial stages – the formation of the two pairs of bright arcs near the SPBs at metaphase II – took place normally in both the wild type and in the *slk1Δ* mutant (Fig. 5A, *t*=0-22 minutes; see Movies 1,2 in the supplementary material). However, growth of the forespore membrane after anaphase II decelerated in the *slk1Δ* mutant (Fig. 5A,B, *t*=24-40 minutes), indicating that the expansion of the forespore membrane after anaphase II does not take place in the absence of Slk1 and, as a consequence, that the forespore membrane closes with a smaller size, resulting in small spores.

Slk1 is localized at the spindle pole body and the forespore membrane

In order to examine the role of Slk1 in the formation of the forespore membrane, we looked for genetic interactions with Spo3, a membrane component required for the assembly of the forespore membrane. Cells deleted for *spo3* completely fail to form the forespore membrane (Nakamura et al., 2001). However, the *spo3-S3* mutant formed small spores, similar to the *slk1Δ* mutant (Fig. 6A). Interestingly, the growth of the forespore membrane in *spo3-S3* mutant cells is also severely impaired after anaphase II (Taro Nakamura, personal communication). We constructed a *spo3-S3 slk1Δ* double mutant and found that these cells were unable to form spores (Fig. 6A,B). However, they were able to form forespore membranes that failed to encapsulate the haploid nuclei (Fig. 6C). Similar phenotypes were found in the double mutant *spo3-GFP slk1* deletion, in which the Spo3 function was slightly compromised (Fig. 6A,B,D). These results indicate a genetic interaction between *spo3* and *slk1*.

Slk1 is highly related to Sid2, which is a mobile component of the SIN pathway that transduces the signal from the SPB to the division site to induce actomyosin-ring contraction and septation (Sparks et al., 1999). The SIN proteins Sid1, Cdc7, Sid2 and Mob1 associate with the SPB during meiosis II, when forespore membrane biosynthesis begins (Krapp et al., 2006). We found that Slk1 was also localized to the SPB during metaphase II and anaphase II; after anaphase II, Slk1 was also localized at the forespore membrane (Fig. 7A,B).

Krapp et al. have shown that the SIN pathway plays a key role in spore formation during meiosis (Krapp et al., 2006). In the absence of SIN components, meiosis takes place normally but the nuclei are not encapsulated by the forespore membrane. This phenotype is similar to the one we found for the *spo3-S3 slk1Δ* double mutant (Fig. 6A,C). We also looked for genetic interactions between *sid2* and *slk1* during meiosis. The temperature-sensitive *sid2-250* mutant was able to carry out meiosis and sporulation at 25°C and 34°C, whereas the double mutant *sid2-250 slk1Δ* was completely unable to sporulate, even at 25°C (Fig. 8A,B). A similar genetic interaction was found between *cdc7-24* and *slk1Δ* (Fig. 8A,B), indicating that Slk1 is absolutely required for spore formation when the SIN pathway is slightly compromised by a temperature-sensitive mutation. In addition, we observed that, in some *sid2-250 slk1Δ* cells, the forespore membrane cut the nucleus, resulting in several masses of DNA (Fig. 8C,D, arrows). Interestingly, the SIN pathway activated normally in the *slk1Δ* mutant, because Sid1 and Cdc7 recruitment to the SPBs, which

signals SIN activation, occurs at meiosis II in *slk1Δ* as in the wild type (Krapp et al., 2006) (see Fig. S2 in the supplementary material). These results indicate that Slk1 and Sid2 protein kinases are the final output of the SIN pathway in meiosis and are required for the correct engulfment of the haploid nuclei by the forespore membrane.

Sid2 overexpression rescues the phenotype of the *slk1Δ* mutant

Sid2 and Slk1 are highly related protein kinases that might perform redundant functions in meiosis. To test whether they were functional paralogues, we overexpressed the *sid2* gene under the control of the *nmt1* promoter (*P41nmt1* version) under repressed

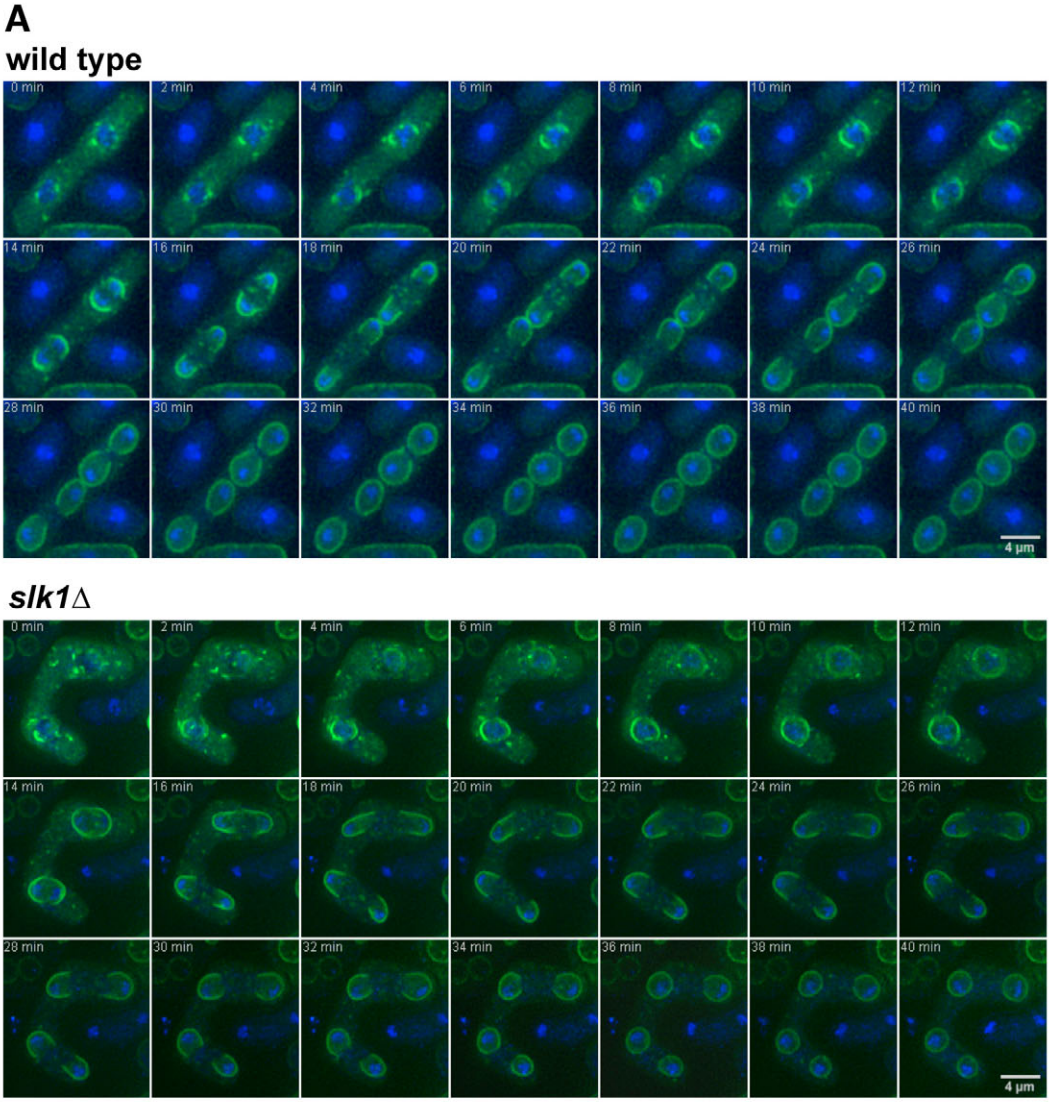

Fig. 5. Forespore membrane growth is defective in the *slk1Δ* mutant. (A) Time-lapse experiments showing forespore membrane growth in wild-type (S1478) and *slk1Δ* (S1883) strains carrying the plasmid pREP81-Psy1-GFP. Cells were sporulated on MEA plates and DNA was stained with Hoechst (blue). Stacks of eight images separated by 0.5 µm were taken every 2 minutes. Deconvolution of images and maximal projections were obtained using Deltavision software. GFP and Hoechst merged images were generated with ImageJ. Scale bars: 4 µm. (B) Forespore membrane (FSM) growth stops prematurely in *slk1Δ* cells. The FSM length of wild-type and *slk1Δ* spores shown in A was measured, and means and standard deviations are represented.

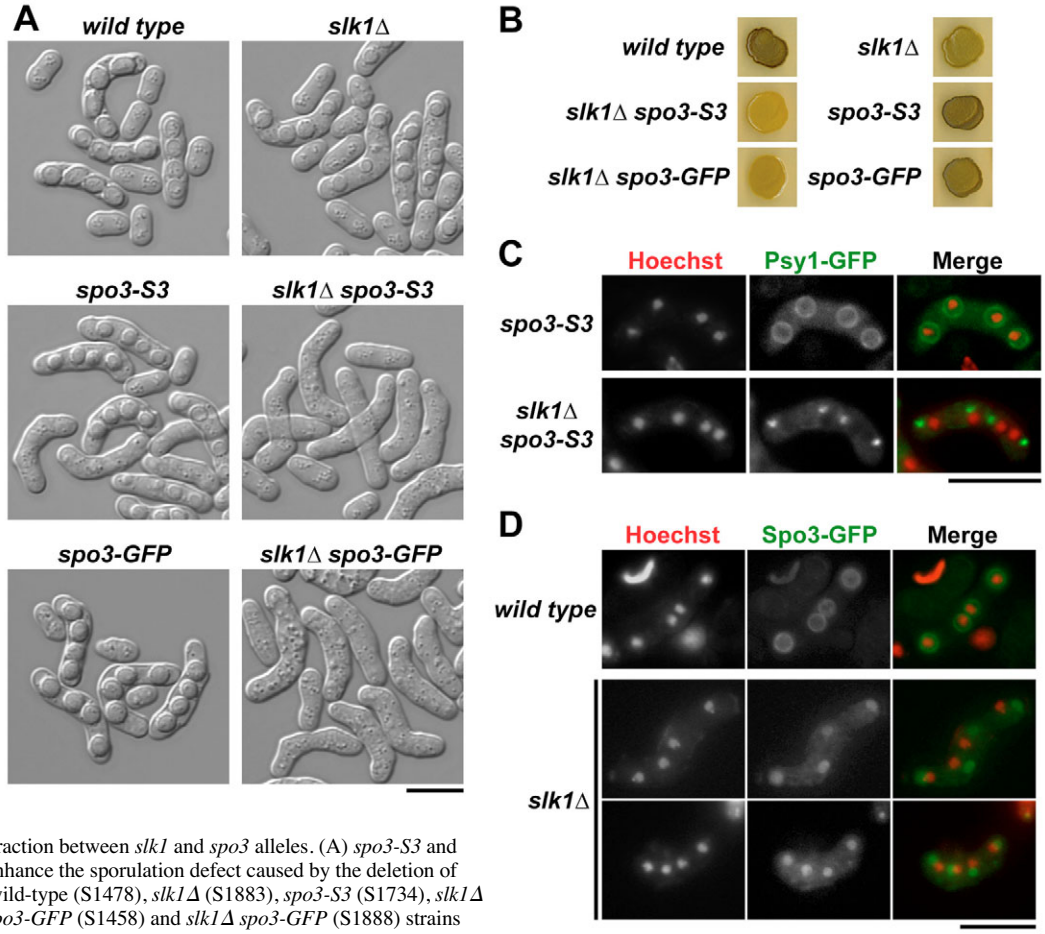

Fig. 6. Genetic interaction between *slk1* and *spo3* alleles. (A) *spo3-S3* and *spo3-GFP* alleles enhance the sporulation defect caused by the deletion of *slk1*. Homothallic wild-type (S1478), *slk1Δ* (S1883), *spo3-S3* (S1734), *slk1Δ spo3-S3* (S1889), *spo3-GFP* (S1458) and *slk1Δ spo3-GFP* (S1888) strains were sporulated on MEA plates, and DIC images were taken after 2 days of incubation at 25°C. (B) MEA plates from the experiment shown in A were stained with iodine vapour after incubation at 25°C for 3 days. Staining is slightly reduced in the *slk1Δ* single mutant, but it is completely abolished in the double mutants. (C) Psy1-GFP fails to encapsulate the nuclei in *slk1Δ spo3-S3* cells. Mutant cells carrying the plasmid pREP81-Psy1-GFP were sporulated on MEA plates at 25°C, stained with Hoechst and photographed. Merged images are shown in the right column. (D) Forespore membranes are unable to engulf the nuclei in *slk1Δ spo3-GFP* cells. Forespore membranes were visualized by Spo3-GFP fluorescence and DNA was stained with Hoechst. Merged images are shown in the right column. Scale bars: 10 μm.

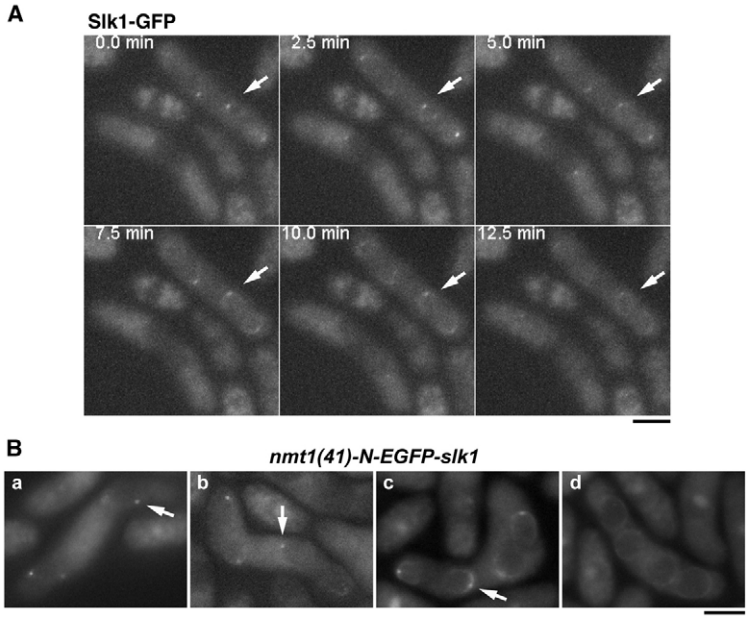

Fig. 7. Slk1-GFP localizes to the spindle pole body and the forespore membrane during meiosis. (A) A homothallic strain harbouring the *slk1*-GFP allele under its own promoter (S1717) was incubated on MEA for 24 hours at 25°C. Meiotic cells were observed under the fluorescence microscope and photographed. Stacks of four images were taken every 2.5 minutes. Deconvolution of images and maximal projections were obtained using Deltavision software. Notice that Slk1 (arrow) moves from the spindle pole body to the forespore membrane (*t*=0-10 minutes). After 12.5 minutes, Slk1-GFP localizes to the forespore membrane. (B) A homothallic strain expressing Slk1-GFP under the *nmt1(41)* promoter (S1931) was incubated on MEA for 24 hours at 25°C and photographed. (Ba) Slk1 (arrow) localizes to the spindle pole body in metaphase II cells. (Bb, Bc) At anaphase II, Slk1 extends from the spindle pole body to the forespore membrane. (Bd) After anaphase II, Slk1 localizes to the forespore membrane. Note that, in the mitotic cells shown in the fields photographed, Slk1 localizes to the nucleolus when overexpressed. Scale bars: 4 μm.

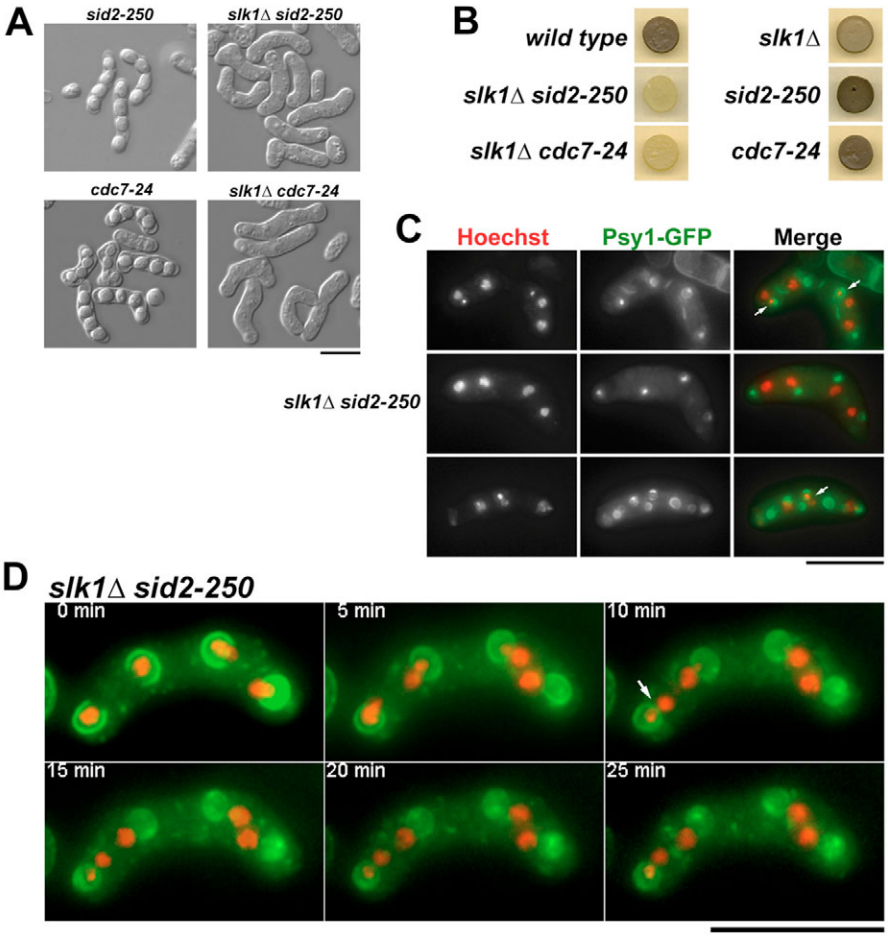

Fig. 8. Genetic interactions between *slk1* and SIN mutants. (A) *sid2-250* and *cdc7-24* alleles enhance the sporulation defect caused by the deletion of *slk1*. Homothallic *sid2-250* (S1884), *cdc7-24* (S1886), *slk1Δ sid2-250* (S1885) and *slk1Δ cdc7-24* (S1887) strains were sporulated on MEA plates, and DIC images were taken after 2 days of incubation at 25°C. Note that double mutants fail to sporulate at the permissive temperature. (B) MEA plates from the experiment shown in A were stained with iodine vapour and photographed. (C) In *slk1Δ sid2-250* cells, forespore membrane growth around the nuclei is defective. h^{90} *slk1Δ sid2-250* cells carrying the plasmid pREP81-Psy1-GFP were sporulated on MEA plates for 2 days at 25°C, stained with Hoechst and photographed. Arrows highlight the abnormal distribution of DNA. (D) Time-lapse experiment showing forespore membrane growth in a *sid2-250 slk1Δ* strain (S1885) carrying plasmid pREP81-Psy1-GFP. Cells were sporulated on MEA plates and DNA was stained with Hoechst. Stacks of five images separated by 1 μm were taken every 5 minutes. GFP (green) and Hoechst (red) merged images were generated with ImageJ. The arrow marks a nucleus that is being cut by the forespore membrane upon closure. Scale bars: 10 μm.

(+thiamine) or derepressed (–thiamine) conditions; *sid2* overexpression was able to fully rescue the *slk1Δ* phenotype (Fig. 9). This result indicates that Sid2 and Slk1 are functionally redundant in meiosis. Interestingly, when a weaker version of the *nmt1* promoter was used (*P81nmt1*), nearly no asci were formed under repressed conditions, resembling the phenotype of the *slk1Δ sid2-250* double mutant (data not shown). Expression of *slk1* in the mitotic cell cycle was unable to rescue the cytokinesis defect of the *sid2-250* mutant (Fig. S3 in the supplementary material). Similarly, *slk1* expression was unable to rescue the morphogenesis defects of the *orb6-25* mutant, which is defective in the Sid2-related kinase Orb6 (Fig. 1B,C and Fig. S3 in the supplementary material). Additionally, according to the meiosis-specific pattern of expression of *slk1* (Fig. 2A,B), no apparent additive defects were observed in the double mutants *slk1Δ sid2-250* and *slk1Δ orb6-25* in cytokinesis or in the control of cell polarity, respectively (Fig. S1 in the supplementary material). These results indicate that, in spite of the high homology between members of the Ndr group of kinases (Fig. 1B,C), Slk1 is unable to perform the functions of related kinases when it is ectopically expressed, suggesting that the function of Slk1 is highly meiosis-specific.

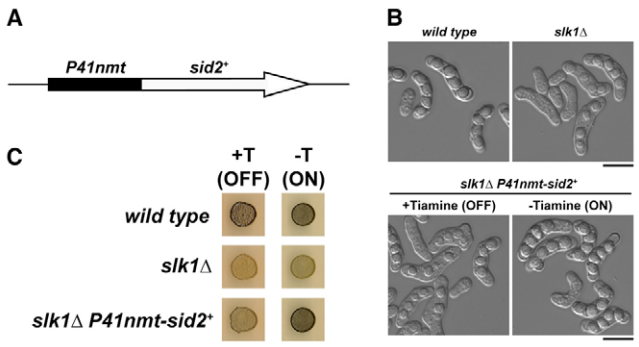

Fig. 9. *sid2*$^{+}$ overexpression rescues the sporulation defect caused by deletion of *slk1*. (A) Schematic representation of the *P41nmt1-sid2* construction. Endogenous *sid2* was under the control of the *P41nmt1* promoter, repressed by thiamine. (B) Homothallic strain *slk1Δ P41nmt1-sid2* (S1890) was spotted onto MEA (*sid2* expression high) and MEA+thiamine (*sid2* expression low). DIC images of asci were taken after 2 days of incubation at 25°C. As controls, images of wild-type (S1478) and *slk1Δ* (S1883) asci are shown. Note that *sid2*$^{+}$ overexpression improves the sporulation of *slk1Δ*, but *sid2* shut-off does not enhance the sporulation defect of the *slk1Δ* mutant, indicating that *sid2* is still expressed in the presence of thiamine. Scale bars: 10 μm. (C) The MEA plates of the experiment shown in B were stained with iodine vapour after 2 days of incubation at 25°C.

Discussion

Role of Slk1 in sporulation

The de novo biosynthesis of the plasma membrane of prespores within the cytoplasm of the mother cell is one of the most intriguing features of sporulation. In mitotic cell division, the plasma membrane of the daughter cells is produced by an extension of the plasma membrane of the mother cell. By contrast, in meiosis, the plasma membrane precursor of the spore is assembled by de novo

biosynthesis. Here, we show that Slk1 plays an important role in spore formation. The *slk1Δ* mutant forms small spores owing to a defect in the degree of extension of the forespore membrane. A similar phenotype has been described for mutants defective in components of the machinery of membrane trafficking and vesicle fusion (Nakamura-Kubo et al., 2003; Nakase et al., 2001). We also observed an additive defect when the deletion of *slk1* was combined with a mutation in *spo3*. Although the exact molecular function of Spo3 is not known, Spo3 is a component of the forespore membrane that is essential for its assembly and stability, and it probably acts in collaboration with the t-SNARE protein Psy1 and the SNAP-25 protein Sec9 (Nakamura et al., 2005; Nakamura et al., 2001). Our experiments indicate that forespore membrane development initiates normally in the four haploid nuclei but decelerates after anaphase II. A similar phenotype has been observed in the *spo3-S3* mutant (T. Nakamura, personal communication), suggesting that both Slk1 and Spo3 might participate in the same step during sporulation (i.e. vesicle fusion and/or forespore membrane stability). Membrane trafficking could be defective in *slk1*-deleted cells, with a smaller amount of membrane reaching the forespore membrane, causing its slower growth. It is noteworthy that, during septation, the redirection of endocytic vesicles to the division site requires a functional SIN pathway (Gachet and Hyams, 2005). In addition, several lines of evidence in higher eukaryotes suggest that SIN-MEN (mitotic exit network)-related proteins regulate the localization of SNARE complexes at the site of cell division. First, human centriolin, which shares homology to the fission yeast Cdc11 and the budding yeast Nud1, is required for exocyst and SNARE-complex localization at the site of abscission, and its disruption causes defects in cytokinesis (Gromley et al., 2005). Second, in *Xenopus*, upregulation of Cdc14A, which is homologous to the downstream component of the SIN-MEN pathway in yeast, Flp1/Cdc14, prevents targeting of the exocyst and SNARE complexes to the midbody (Krasinska et al., 2007).

How does the SIN-MEN pathway regulate membrane trafficking during septation and sporulation? Much research has been directed towards uncovering the substrates of the most downstream kinases of the SIN-MEN pathway (i.e. Sid2-Mob1 in fission yeast, and Dbf2-Mob1 in budding yeast). Interestingly, protein analyses aimed at identifying in vitro substrates of Dbf2-Mob1 yielded, among others, a protein involved in the endocytic pathway (Vps27/Sst4), suggesting that Dbf2 might phosphorylate proteins involved in endocytosis and protein sorting (Mah et al., 2005). This could be the case for Slk1 during sporulation. Alternatively, Slk1 could phosphorylate meiosis-specific proteins involved in forespore membrane growth, such as Spo3, or proteins at the leading edge of the forespore membrane, such as Meu14 (Okuzaki et al., 2003). Although Meu14 was recruited to the leading edge of the forespore membrane in the *slk1Δ* mutant as in the wild type (data not shown), the leading-edge complex plays an important role in the growth and shaping of the prospore membrane in *S. cerevisiae* and, therefore, its defective regulation might be behind the abnormal growth of spores in the *slk1Δ* mutant (Moreno-Borchart et al., 2001; Neiman, 2005). These analyses will be addressed in future studies and could shed light on targets of the SIN pathway during sporulation and septation in fission yeast, as well as on the process of abscission in higher eukaryotes.

Another possibility is that *slk1Δ* could be defective in coordinating the exit from meiosis II with sporulation. This is the case of the budding yeast *CDC15* (homologous to *cdc7* in fission yeast), mutants of which result in defects in the disassembly of anaphase II spindles and of the meiotic outer plaque of SPBs, leading to a sporulation defect similar to that described in SIN mutants; that is, the inability of forespore membranes to properly engulf haploid nuclei (Pablo-Hernando et al., 2007). Interestingly, the function of Cdc15 in sporulation seems to be independent of MEN and Cdc14 functions in meiotic divisions. In fission yeast, *slk1Δ*, similar to other SIN mutants, is not defective in meiotic divisions nor in the assembly and disassembly of the meiotic spindles (Krapp et al., 2006) (Fig. S4 in the supplementary material). However, because a certain redundancy exists between Sid2 and Slk1 in sporulation (see below), spindle and SPB dynamics should be analyzed in double mutants in order to avoid any compensation effects. It is also interesting to note that the severe segregation defects observed in *slk1Δ sid2-250* and *slk1Δ cdc7-24* mutants, in which about 50% of the asci contained more than four DAPI-stained bodies (Fig. 8C,D, and L.P.-H. and S.M., unpublished observations), seem to be due to the aberrant dynamics of forespore membrane biosynthesis. In some cases, uncoordinated growth and closure of the forespore membrane resulted in a meiotic 'cut' phenotype (Fig. 4C, Fig. 8C,D). 'Cut' nuclei were less frequent in the double mutant *slk1Δ spo3-S3*, which does not form spores, suggesting that, in double mutants *slk1Δ sid2-250* and *slk1Δ cdc7-24*, forespore membrane defects are more severe.

Slk1: a meiotic Sid2 paralogue

Slk1 and Sid2 perform redundant functions in sporulation, as shown by the fact that the thermosensitive allele of *sid2*, *sid2-250*, increases the sporulation defect of *slk1Δ* even at the permissive temperature. Conversely, increased expression of *sid2* in meiosis suppresses the sporulation defect of *slk1Δ*. The role of Sid2 in sporulation has not been shown until now because available *sid2* mutants do not have apparent sporulation defects. The existence of a meiosis-specific Sid2-like kinase in fission yeast explains the lack of sporulation phenotype of *sid2* alleles. In budding yeast, a function of the MEN downstream kinases Dbf2 and Dbf20 in sporulation has not been reported. Blast searches failed to reveal the existence of a meiosis-specific homologue. However, a role for Dbf2 and/or Dbf20 in sporulation cannot be ruled out, because some MEN components have been shown to play a role in spore morphogenesis (Gordon et al., 2006; Kamieniecki et al., 2005; Pablo-Hernando et al., 2007). Dbf20 expression peaks later than Dbf2 during meiosis, suggesting that Dbf20 could be a better candidate to perform a role in sporulation than Dbf2 (Chu et al., 1998).

Two additional intriguing aspects connect Slk1 with Sid2 and the SIN pathway. First, during the mitotic cycle, Sid2 initially localizes to the SPBs and moves to the medial ring upon SIN activation. Similarly, Slk1 first localizes to the SPBs and then moves to the forespore membrane. It remains to be tested what the signals are that regulate this translocation. Second, in the *slk1Δ* mutant, the forespore membrane growth decelerates concurrently with the onset of anaphase B and Cdc2 inactivation (Fig. 5), coinciding in time with SIN activation in meiosis (Krapp et al., 2006). Given the conservation of the Sid2 family of protein kinases in mammalian cells, it will be worth studying the function of these proteins in cytokinesis and membrane biosynthesis at the end of mitosis and meiosis.

Materials and Methods

Yeast strains and methods

Strains used in this study are listed in Table S1 in the supplementary material. Fission yeast cells were grown and manipulated according to standard protocols (Moreno et al., 1991). Genetic crosses were done on malt extract agar plates (MEA). Cells were grown in yeast extract with supplements (YES) or Edinburgh minimal medium (EMM)

at the appropriate temperatures. To repress expression from the *nmt1* promoter, 5 μg/ml of thiamine was added to minimal medium.

Diploid *pat1-114* strains were generated by protoplast fusion (Sipiczki and Ferenczy, 1977). Meiotic time-course experiments were done as described previously (Blanco et al., 2001). Briefly, *pat1-114* diploid cells were grown in YES until the exponential phase and then transferred to EMM supplemented with leucine at 100 μg/ml. At late exponential phase, cells were washed and transferred to EMM-N (+leucine at 50 μg/ml). After 14 hours, cells were induced to enter meiosis by shifting the temperature to 34°C. Nitrogen was reintroduced (0.5 g/l of NH_4Cl) and an additional supplement of leucine was added (50 μg/ml).

Slk1 GFP-tagging and *P41nmt-sid2* construction

Slk1 was C-terminally tagged with the GFP epitope using the PCR-based method described previously (Bahler et al., 1998). Oligonucleotides with 80 bases of homology to regions flanking the *slk1* stop codon were used to amplify the GFP and *kanMX6* sequence from plasmid *pFA6a-GFP-kanMX6*. This PCR product was used to transform fission yeast cells. Transformation was performed following the lithium acetate protocol. Correct GFP integration was checked by PCR. A similar protocol was followed to insert the *P41nmt1* promoter at the *sid2* locus, in this case with oligonucleotides with homology to regions around the *sid2* initiation codon.

Construction of *slk1*-containing plasmids

The *slk1*$^+$ cDNA was amplified by PCR using cDNA obtained from a 4-hour *pat1* meiotic culture with primers slk1-N 5′-TTTT*CTCGAG*GATCCCATGGACCTA-CTGGGCCTTAAAG-3′ (*Bam*HI site, underlined; *Xho*I site, italicized) and slk1-C 5′-TTTTCCCGGG*TTA*GAGCAAAAATTCATACAGGTC-3′ (*Sma*I site, underlined; an added stop codon, italicized). This PCR product was digested with *Xho*I and *Sma*I and cloned into a similarly digested pREP3X vector (Forsburg, 1993), producing plasmid pREP3X-*slk1*$^+$, which contains *slk1*$^+$ cDNA under the *nmt1(3X)* promoter. Plasmid pREP41-N-EGFP-*slk1*$^+$, which expresses *slk1*$^+$ N-terminally tagged with EGFP from the *nmt1(41)* promoter, was constructed by cloning the *Bam*HI-*Sma*I fragment from pREP3X-*slk1*$^+$, containing *slk1*$^+$, into the same sites of pREP41-N-EGFP (Craven et al., 1998). The *Pst*I-*Eco*RI fragment from pREP41-N-EGFP-*slk1*$^+$, containing *nmt1(41)-EGFP-slk1*$^+$, was cloned into the same sites of the integrative vector pJK148, generating plasmid pJK148-P41nmt1-EGFP-*slk1*$^+$ (Keeney and Boeke, 1994).

To generate strain S1931, carrying an integrated version of GFP-*slk1* under the *nmt1(41)* promoter, plasmid pJK148-P41nmt1-EGFP-*slk1*$^+$ was linearized with *Nru*I and integrated into the *S. pombe leu1* locus by homologous recombination. Correct integration was checked by Southern blot analysis.

RNA and protein methods

RNA was extracted by the phenol-chloroform method (Moreno et al., 1991). 8 μg of total RNA were run on agarose gels in the presence of formaldehyde. RNA was blotted onto GeneScreen Plus membranes (NEN, Dupont) and hybridized with a radioactively labelled probe covering the entire open reading frame (ORF) of the *slk1* cDNA.

Total protein extracts were made using the trichloroacetic acid (TCA) extraction protocol (Foiani et al., 1994). Protein extracts were run on 8% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). For Slk1-GFP detection, mouse monoclonal anti-GFP (Living Colors, Clontech) was used as the primary antibody (1:1000 dilution). Tubulin was detected using mouse monoclonal anti-TAT1 antibodies (1:3000 dilution). Goat anti-mouse conjugated to horseradish peroxidase (Amersham) was used as the secondary antibody at 1:2500 dilution. Membranes were developed with Supersignal (Pierce) or ECL western blotting reagents (Amersham).

Flow cytometry

Ethanol-fixed cells were stained with propidium iodine as described previously (Moreno et al., 1991; Sazer and Sherwood, 1990). Samples were analyzed using a Becton-Dickinson FACScan flow cytometer equipped with CellQuest software.

Microscopy

For DAPI staining of nuclei, cells were fixed with 70% ethanol, washed in PBS and resuspended in PBS plus 1 μg/ml DAPI. Nucleus staining of live cells was performed with Hoechst at 1-2 μg/ml in EMM or PBS. For calcofluor staining, ethanol-fixed cells were washed in PBS and resuspended in PBS with calcofluor at a final concentration of 50 μg/ml.

Time-lapse experiments shown in Fig. 5 and supplementary material Fig. S4 were performed using a Deltavision RT deconvolution microscope system (Applied Precision, Issaquah, WA) equipped with an Olympus IX71 microscope and a CoolSNAP HQ camera (Photometrics). Time-lapse experiments shown in Fig. 4C and Fig. 8D were performed with a Nikon Eclipse E2000 fluorescence microscope coupled to a Hamamatsu camera, and equipped with MetaMorph software. Other images were acquired with a Leica or a Zeiss Axioplan2 microscope coupled to Hamamatsu cameras and equipped with Openlab software (Improvision), or with a Nikon Eclipse E2000 fluorescence microscope using Metamorph software. Images were processed and assembled with Adobe Photoshop and ImageJ software.

We thank members of our laboratory for discussions during the course of this work; Viesturs Simanis, Pilar Pérez and the Yeast Genetic Resource Center (YGRC, Japan) for plasmids and strains; Rafa Daga for his help with the Deltavison microscope and comments on the manuscript; and Taro Nakamura for sharing with us unpublished results. This work was supported by grants from the Spanish Ministry of Science and Education to S.M. (BFU2005-03195, GEN2003-20243-C08-05 and CDS2007-00015).

References

Bahler, J., Schuchert, P., Grimm, C. and Kohli, J. (1991). Synchronized meiosis and recombination in fission yeast: observations with pat1-114 diploid cells. *Curr. Genet.* **19**, 445-451.

Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast* **14**, 943-951.

Blanco, M. A., Pelloquin, L. and Moreno, S. (2001). Fission yeast mfr1 activates APC and coordinates meiotic nuclear division with sporulation. *J. Cell Sci.* **114**, 2135-2143.

Chang, L. and Gould, K. L. (2000). Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast. *Proc. Natl. Acad. Sci. USA* **97**, 5249-5254.

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O. and Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. *Science* **282**, 699-705.

Craven, R. A., Griffiths, D. J., Sheldrick, K. S., Randall, R. E., Hagan, I. M. and Carr, A. M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* **221**, 59-68.

Fankhauser, C. and Simanis, V. (1994). The cdc7 protein kinase is a dosage dependent regulator of septum formation in fission yeast. *EMBO J.* **13**, 3011-3019.

Foiani, M., Marini, F., Gamba, D., Lucchini, G. and Plevani, P. (1994). The B subunit of the DNA polymerase alpha-primase complex in Saccharomyces cerevisiae executes an essential function at the initial stage of DNA replication. *Mol. Cell. Biol.* **14**, 923-933.

Forsburg, S. L. (1993). Comparison of *Schizosaccharomyces pombe* expression systems. *Nucleic Acids Res.* **21**, 2955-2956.

Gachet, Y. and Hyams, J. S. (2005). Endocytosis in fission yeast is spatially associated with the actin cytoskeleton during polarised cell growth and cytokinesis. *J. Cell Sci.* **118**, 4231-4242.

Gordon, O., Taxis, C., Keller, P. J., Benjak, A., Stelzer, E. H., Simchen, G. and Knop, M. (2006). Nud1p, the yeast homolog of Centriolin, regulates spindle pole body inheritance in meiosis. *EMBO J.* **25**, 3856-3868.

Gromley, A., Yeaman, C., Rosa, J., Redick, S., Chen, C. T., Mirabelle, S., Guha, M., Sillibourne, J. and Doxsey, S. J. (2005). Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. *Cell* **123**, 75-87.

Guertin, D. A., Chang, L., Irshad, F., Gould, K. L. and McCollum, D. (2000). The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. *EMBO J.* **19**, 1803-1815.

Hagan, I. and Yanagida, M. (1995). The product of the spindle formation gene sad1+ associates with the fission yeast spindle pole body and is essential for viability. *J. Cell Biol.* **129**, 1033-1047.

Hirata, A. and Shimoda, C. (1994). Structural modification of spindle pole bodies during meiosis II is essential for the normal formation of ascospores in Schizosaccharomyces pombe: ultrastructural analysis of spo mutants. *Yeast* **10**, 173-183.

Hou, M. C., Salek, J. and McCollum, D. (2000). Mob1p interacts with the Sid2p kinase and is required for cytokinesis in fission yeast. *Curr. Biol.* **10**, 619-622.

Kamieniecki, R. J., Liu, L. and Dawson, D. S. (2005). FEAR but not MEN genes are required for exit from meiosis I. *Cell Cycle* **4**, 1093-1098.

Keeney, J. B. and Boeke, J. D. (1994). Efficient targeted integration at leu1-32 and ura4-294 in *Schizosaccharomyces pombe*. *Genetics* **136**, 849-856.

Krapp, A., Schmidt, S., Cano, E. and Simanis, V. (2001). S. pombe cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body. *Curr. Biol.* **11**, 1559-1568.

Krapp, A., Collin, P., Cokoja, A., Dischinger, S., Cano, E. and Simanis, V. (2006). The Schizosaccharomyces pombe septation initiation network (SIN) is required for spore formation in meiosis. *J. Cell Sci.* **119**, 2882-2891.

Krasinska, L., de Bettignies, G., Fisher, D., Abrieu, A., Fesquet, D. and Morin, N. (2007). Regulation of multiple cell cycle events by Cdc14 homologues in vertebrates. *Exp. Cell Res.* **313**, 1225-1239.

Mah, A. S., Elia, A. E., Devgan, G., Ptacek, J., Schutkowski, M., Snyder, M., Yaffe, M. B. and Deshaies, R. J. (2005). Substrate specificity analysis of protein kinase complex Dbf2-Mob1 by peptide library and proteome array screening. *BMC Biochem.* **6**, 22.

Martin-Castellanos, C., Blanco, M., Rozalen, A. E., Perez-Hidalgo, L., Garcia, A. I., Conde, F., Mata, J., Ellermeier, C., Davis, L., San-Segundo, P. et al. (2005). A large-scale screen in S. pombe identifies seven novel genes required for critical meiotic events. *Curr. Biol.* **15**, 2056-2062.

Mata, J., Lyne, R., Burns, G. and Bahler, J. (2002). The transcriptional program of meiosis and sporulation in fission yeast. *Nat. Genet.* **32**, 143-147.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. *Meth. Enzymol.* **194**, 795-823.

Moreno-Borchart, A. C., Strasser, K., Finkbeiner, M. G., Shevchenko, A., Shevchenko, A. and Knop, M. (2001). Prospore membrane formation linked to the leading edge protein (LEP) coat assembly. *EMBO J.* **20**, 6946-6957.

Nakamura, T., Nakamura-Kubo, M., Hirata, A. and Shimoda, C. (2001). The Schizosaccharomyces pombe spo3+ gene is required for assembly of the forespore membrane and genetically interacts with psy1(+)-encoding syntaxin-like protein. *Mol. Biol. Cell* **12**, 3955-3972.

Nakamura, T., Kashiwazaki, J. and Shimoda, C. (2005). A fission yeast SNAP-25 homologue, SpSec9, is essential for cytokinesis and sporulation. *Cell Struct. Funct.* **30**, 15-24.

Nakamura-Kubo, M., Nakamura, T., Hirata, A. and Shimoda, C. (2003). The fission yeast spo14+ gene encoding a functional homologue of budding yeast Sec12 is required for the development of forespore membranes. *Mol. Biol. Cell* **14**, 1109-1124.

Nakase, Y., Nakamura, T., Hirata, A., Routt, S. M., Skinner, H. B., Bankaitis, V. A. and Shimoda, C. (2001). The Schizosaccharomyces pombe spo20(+) gene encoding a homologue of Saccharomyces cerevisiae Sec14 plays an important role in forespore membrane formation. *Mol. Biol. Cell* **12**, 901-917.

Neiman, A. M. (2005). Ascospore formation in the yeast Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* **69**, 565-584.

Okuzaki, D., Satake, W., Hirata, A. and Nojima, H. (2003). Fission yeast meu14+ is required for proper nuclear division and accurate forespore membrane formation during meiosis II. *J. Cell Sci.* **116**, 2721-2735.

Pablo-Hernando, M. E., Arnaiz-Pita, Y., Nakanishi, H., Dawson, D., Del Rey, F., Neiman, A. M. and de Aldana, C. R. (2007). Cdc15 is required for spore morphogenesis independently of Cdc14 in Saccharomyces cerevisiae. *Genetics* **177**, 281-293.

Salimova, E., Sohrmann, M., Fournier, N. and Simanis, V. (2000). The S. pombe orthologue of the S. cerevisiae mob1 gene is essential and functions in signalling the onset of septum formation. *J. Cell Sci.* **113**, 1695-1704.

Sazer, S. and Sherwood, S. W. (1990). Mitochondrial growth and DNA synthesis occur in the absence of nuclear DNA replication in fission yeast. *J. Cell Sci.* **97**, 509-516.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A. and Simanis, V. (1997). The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in Schizosaccharomyces pombe. *Genes Dev.* **11**, 1519-1534.

Shimoda, C. (2004). Forespore membrane assembly in yeast: coordinating SPBs and membrane trafficking. *J. Cell Sci.* **117**, 389-396.

Shimoda, C. and Nakamura, T. (2004). Control of late meiosis and ascospore formation. In *The Molecular Biology of Schizosaccharomyces pombe: Genetics, Genomics and Beyond* (ed. R. Egel), pp. 311-327. Berlin, Heidelberg, New York: Springer-Verlag.

Sipiczki, M. and Ferenczy, L. (1977). Protoplast fusion of Schizosaccharomyces pombe Auxotrophic mutants of identical mating-type. *Mol. Gen. Genet.* **151**, 77-81.

Sparks, C. A., Morphew, M. and McCollum, D. (1999). Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *J. Cell Biol.* **146**, 777-790.

Tamaskovic, R., Bichsel, S. J. and Hemmings, B. A. (2003). NDR family of AGC kinases-essential regulators of the cell cycle and morphogenesis. *FEBS Lett.* **546**, 73-80.

Tanaka, K. and Hirata, A. (1982). Ascospore development in the fission yeasts *Schizosaccharomyces pombe* and *S. japonicus*. *J. Cell Sci.* **56**, 263-279.

Tomlin, G. C., Morrell, J. L. and Gould, K. L. (2002). The spindle pole body protein Cdc11p links Sid4p to the fission yeast septation initiation network. *Mol. Biol. Cell* **13**, 1203-1214.